Regulation of Blastocyst Migration, Apposition, and Initial Adhesion by a Chemokine, Interferon γ-inducible Protein 10 kDa (IP-10), during Early Gestation*

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For a pregnancy to be established, initial apposition and adhesion of the blastocyst to maternal endometrium must occur in a coordinated manner; however, a key factor(s) that mediates the trophoblast cell migration and attachment to the apical surface of the endometrium has not been identified. In this study, we examined the effect of an endometrial chemokine, interferon-γ-inducible protein 10 kDa (IP-10), on conceptus migration to the endometrial epithelium. We first studied endometrial IP-10 mRNA expression, which was localized in the subepithelial stromal region, and detected the protein in the uterine flushing media during early pregnancy. Expression of IP-10 mRNA by the endometrium of cyclic animals was stimulated by the addition of a conceptus factor interferon-tau (IFN-τ). Immunofluorescent analysis revealed that IP-10 receptor, CXCR3, was localized in the trophoblast cells, to which biotinylated-recombinant caprine IP-10 (rcIP-10) bound. Chemotaxis assay indicated that rcIP-10 stimulated the migration of trophoblast cells, and the effects of rcIP-10 were neutralized by the pretreatment with an anti-IP-10 antibody. Adhesive activity of trophoblast cells to fibronectin was promoted by rcIP-10, and the effect was inhibited by the use of anti-IP-10 antibody. Further adhesion experiments demonstrated that binding of trophoblast cells to fibronectin was completely inhibited by a peptide of the Arg-Gly-Asp (RGD) sequence, which binds to integrins $\alpha_5\beta_1$, $\alpha_V\beta_1$, $\alpha_V\beta_3$, and $\alpha_V\beta_5$, whereas non-binding peptide containing Arg-Gly-Glu (RGE) had minimal effects. More importantly, rcIP-10 promoted the adhesion of trophoblast cells to primary cells isolated from endometrial epithelium. Furthermore, rcIP-10 stimulated the expression of integrin α_5, α_V, and β_3 subunit mRNA in trophoblast cells. These findings suggest that endometrial IP-10 regulates the establishment of apical interactions between trophoblast and epithelial cells during early gestation.

Implantation requires closely harmonized processes of apposition, attachment, and adhesion of the conceptus to the maternal endometrium and is completed when an initial formation of the placenta between the conceptus and endometrium is established. In the ruminant ungulates, interferon-τ (IFN-τ),[1] secreted by the trophoblast cells, plays a role in the process of maternal recognition of pregnancy (1, 2). When IFN-τ production is highest between days 15 and 18 of gestation, the elongated conceptus reaches a point of attachment *in utero*, and fuses with endometrial epithelial cells, which undergoes the developmental changes, resulting in the formation of a noninvasive synepitheliochorial placenta (3). When the conceptus attachment begins, physical interactions are established between the tips of the endometrial microvilli and the trophoblast cells, which involve changes in the expression of cell adhesion molecules and extracellular matrix (ECM) proteins (4–6). Integrins are a large family of cell surface receptors that mediate the attachment of cells possessing ECM. The expression of integrins on endometrial luminal epithelium changes during the estrous cycle, and such expressions on uterine surface and embryonic trophectoderm also change during gestation. In addition, the increase in integrin expressions on the trophectoderm during the early pregnancy is required for initial and/or firm adhesion of the conceptus to endometrial cells. However, a key factor(s) that mediates the trophoblast cell migration and attachment to the apical surface of endometrium has not been identified.

Recently, we demonstrated that a chemokine, interferon-γ-inducible protein 10 kDa (IP-10), was expressed in the pregnant ovine endometrium, and the endometrial tissue expression of IP-10 *in vitro* was stimulated by the addition of conceptus factor IFN-τ (7). Chemokines are a family of structurally related low molecular mass cytokines and play a key role in the migration and activation of different subpopulations of leukocytes (8). Four subfamilies, designated C, CC, CXC, and CX_3C chemokines, are identified based on the relative positions of conserved cysteine residues (9). IP-10, belongs to the CXC chemokine subfamily, is induced in a variety of cell types (10), and regulates multiple aspects of inflammatory and immune responses primarily through chemotactic activity toward subsets of leukocytes (11). IP-10 targets the receptor CXCR3 and activates several subsets of leukocytes, NK cells, Th1 lymphocytes, eosinophils and dendritic cells (12–15). Several chemo-

[1] The abbreviations used are: IFN, interferon; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; ECM, extracellular matrix; LSM, least square means; IP-10, interferon γ-inducible protein 10 kDa.

kines have been discovered in the fetal and maternal annexes during the early pregnancy in humans (16–18), mice (19–21), and ruminants (7, 22, 23). These chemokines are thought to play an important role in cellular movement and positioning of leukocytes, which infiltrate into the subepithelial stromal regions of the uterus. It has been suspected that distribution and/or redistribution of leukocytes, regulated by chemokines expressed *in utero* in a spatial-temporal manner, is necessary for the establishment of immunological environments suitable for implantation and subsequent development.

It is known that non-hematopoietic tumor cells exhibit specific patterns of metastasis to other organs, suggesting that tumor cells do not migrate randomly. One explanation for this phenomenon is that the migration of tumor cells may be determined by interactions between particular chemokines and specific receptors at target organs (24, 25). It has been reported that some chemokine receptor transcripts are detected in human and mouse non-hematopoietic trophoblast cells (26–28). These observations lead us to postulate that interactions between chemokines and their receptors modulate trophoblast migration to the endometrium. Moreover, chemokines may stimulate the integrin-dependent adhesion of trophoblast cells to the apical surface of endometrium because chemokines are also known as physiological activators of rapid integrin-dependent leukocytes arrest on endothelial cells (29). However, it is unclear whether endometrial IP-10, stimulated by conceptus factor IFN-τ, modulates the migration and adhesion of trophoblast cells to the endometrium in ruminants.

In the present study, we assessed a putative role that IP-10/CXCR3 plays within the uterus during early pregnancy in ruminants. To study a role of IP-10/CXCR3 for engagement of the conceptus and endometrium, temporal expression of IP-10 and CXCR3 within the uterus of Shiba goats was examined. Whether the interaction between IP-10 and CXCR3 could mediate trophoblast cell migration and adhesion to the apical surface of endometrium was then determined. Examination is extended to identify which integrin subunits and ECMs were involved in the trophoblast cell adhesion to the endometrium through the IP-10/CXCR3 signaling.

MATERIALS AND METHODS

Animals and Tissue Preparation—Shiba goats were maintained at the University of Tokyo farm and the protocol for goat experimentation was reviewed and approved by the Animal Care Committee at the university. Uteri from cyclic goats on days 14 (n = 9) and 17 (n = 3) and pregnant goats on days 14 (n = 6), 17 (n = 9), and 20 (n = 3) were removed by hysterectomy under isoflurane anesthesia. Whole uteri from day 14 cyclic and days 14 and 17 pregnant animals (n = 3 each) were frozen for *in situ* hybridization analysis (7). Uteri from days 14 and 17 pregnant and days 14 and 17 cyclic animals were subjected to uterine flushing procedure for the isolation of conceptuses and/or collection of flushed media. PBS (15 ml) was gently flushed into the uterus, and the uterine flushing media collected were concentrated (Macrosep, Pall Corporation, East Hills, NY) and subjected to the Western blot analysis for the detection of IP-10. Conceptus tissues collected from days 14 (n = 3, whole conceptus), 17 (n = 3, approximately one half of conceptus, 200 mg each) and 20 (n = 3, trophoblast region only) of pregnant animals were subjected to *in vitro* culture, the remaining days 17 and 20 conceptus tissues were frozen for subsequent RNA extraction and immunostaining. The *in vitro* culture media were subjected to Western blot analysis for the determination of IFN-τ. Uteri from day 14 cyclic animals (n = 3) were used for epithelial cell isolation. Endometrial explants from day 14 cyclic goats (n = 3) were cultured to examine the stimulatory effect of IFN-τ on IP-10 production. Endometrial tissues of goats (n = 3 for each day examined) from which conceptuses had been removed were frozen and subjected to RNA extraction.

In Vitro Culture—Whole conceptuses (day 14, n = 3) and minced conceptuses (days 17 and 20, ~200 mg/culture dish, n = 3 each) were cultured under conditions essentially described previously (30).

The procedures for endometrial epithelial cell dissociation and subsequent characterization were previously described (31). Uterine horns

TABLE I
Oligonucleotide primers used for PCR reactions

Name	Sequence of foward and reverse primer	Length
		bp
CXCR3	5'-GCATCAGCTTCGATCGGTAC-3'	283
	5'-GATGCGGGCGTAGCAATAGG-3'	
XCR1	5'-ATGGAGCCCTCAGACATCCC-3'	627
	5'-GAGGATCTCCACGTAGCAGA-3'	
Integrin α5	5'-TGCTGTGAACCAGAGTCGTC-3'	809
	5'-ATCCACTGCACAGCTGTGGC-3'	
Integrin αV	5'-GAAGCAGGAAAGAGAGCCTG-3'	890
	5'-CTATATCCGTGGCTCCTTTC-3'	
Integrin β1	5'-CTCAAATCCAGCCACAGCAG-3'	523
	5'-CCAGCGAAGTGAAACACAGC-3'	
Integrin β3	5'-AGATTGGAGACACGGTGAGC-3'	392
	5'-GTACTTGAAAGTGATCTTGC-3'	
Integrin β5	5'-GTCTGAAGATTGGGGACAGC-3'	285
	5'-GGTACACGCTCTGGTTCTCC-3'	
G3PD11	5'-ATGGGGAAGGTGAAGGTCGG-3'	150
	5'-ATCATATTGGAACATGTAAA-3'	

from three day 14 cyclic goats were filled with 0.76% EDTA-PBS and preincubated at 37 °C for 1 h. The endometrial epithelium was scraped off by a surgical blade and then incubated at 37 °C for 8 min in 0.1% collagenase (Sigma). The cell suspension was passed through a nylon mesh (70 μm) and subjected to density gradient centrifugation. Collected epithelial cells were resuspended in Dulbecco's modified Eagle's medium and Ham's F-12 (DMEM/Ham's F-12, Sigma) supplemented with 40 units/ml of penicillin, 40 μg/ml, of streptomycin and 10% fetal calf serum. The cells were then plated in 24-well dishes coated with swine-skin type I collagen (Nitta gelatin, Osaka, Japan) and subjected to the adhesion assay when the cells had grown to confluent.

Dissociated cells were also prepared from day 17 goat conceptuses. These conceptuses (n = 3) were dissected into small pieces and incubated in 0.2% collagenase (Sigma) at 37 °C for 30 min. The cells were passed through a nylon mesh (70 μm) and resuspended in DMEM with antibiotics, which were immediately used for chemotaxis or adhesion assays. In addition, bovine B cells (KU-1, Ref. 32) and caprine trophoblast clone cells (HTS-1, Ref. 33) were cultured in DMEM supplemented with 10–20% fetal calf serum and antibiotics.

Endometrial tissues (~600 mg wet weight/culture dish) from day 14 cyclic goats (n = 3) were cultured in 20 ml of DMEM supplemented with antibiotics, and were treated with 5 nM recombinant bovine IFN-τ (rbIFN-τ, Katakura Industries, Inc., Tokyo, Japan). After 24 h at 37 °C and 5% CO_2 atmosphere, endometrial tissues were frozen separately and stored at −70 °C for subsequent RNA extraction and Northern blot analyses.

Cloning of Caprine IP-10 and CXCR3 cDNAs—Through the use of RT-PCR, caprine IP-10 and CXCR3 cDNAs were amplified from caprine endometrial RNA and subcloned into a pSTBlue vector (TaKaRa, Tokyo, Japan), which was then subjected to an automated sequence analysis using a PerkinElmer sequencer (model ABI Prism 377 XL, Roche Applied Science). Nucleotide sequence comparisons of caprine IP-10 and CXCR3 were performed using the BLAST network program (National Center for Biotechnology Information, National Institutes of Health, Bethesda, MD) and GenBank™ accession numbers were obtained (AB099892 and AB099893, respectively).

Transfection of CXCR3 Expression Plasmid—The nucleotide sequence encoding the open reading frame of caprine CXCR3 was PCR-amplified and cloned into a mammalian expression vector pcDNA3.1 (Invitrogen Corp., Carlsbad, CA). This plasmid was transfected into KU-1 and HTS-1 cells by using Transfast™ (Promega, Madison, WI) according to the manufacturer's protocol. Two days after transfection, cells were used for chemotaxis or adhesion assay.

Production of Recombinant Caprine IP-10 and the Antibody—The nucleotide sequence encoding the mature region of caprine IP-10 (cIP-10) cDNA was PCR-amplified and cloned into an expression plasmid, pET-14b (Novagen, Madison, WI), which consisted of a histidine tag at the N-terminal side of cIP-10. This expression vector, pET-14b-cIP-10, was used to transform *Escherichia coli* BL21-SI (Invitrogen) cells, which were grown overnight. These cells were harvested, resuspended in 50 mM NaH_2PO_4, 500 mM NaCl, and 10 mM imidazole (pH 7.4), and disrupted on ice by sonication. After cell debris and insoluble proteins were removed by centrifugation, recombinant cIP-10 (rcIP-10) in the supernatant was examined using SDS-PAGE. Recombinant cIP-10 was purified using a nickel-chelating column (Hi-trap Chelating HP, Amersham Biosciences) on the chromatography system (AKTA, Amersham

Regulation of Trophoblast Cell Migration and Adhesion by IP-10

FIG. 1. **Preparation of recombinant caprine IP-10 and the antibody.** *A*, recombinant caprine IP-10 (rcIP-10) was expressed in *E. coli* BL21-SI, and cell lysates before (*lane 1*) and after (*lane 2*) purification using a nickel-chelating column was subjected to SDS-PAGE. *B*, purified rcIP-10 protein (50 or 200 ng/lane) was subjected to Western blot analysis with either anti-histidine tag (*lane 1*) or anti-caprine IP-10 antibody (30 μg/ml) generated in our laboratory (*lane 2*). *C*, *left*, Northern blot analysis of CXCR3 mRNA. RNA was extracted from KU-1 cells that had been transiently transfected with pcDNA3.1-caprine CXCR3 (*CXCR3*) or parental pcDNA3.1 (*Mock*). *Right*, biological activity of rcIP-10 to CXCR3 transfected KU-1 cells expressing (*circle*) or not expressing (*square*) CXCR3 was tested by chemotaxis assay. *Bars* represent LSM ± S.E. *D*, chemotaxis activity was demonstrated in KU-1 cells with the addition of 20 ng/ml rcIP-10, to which no further treatment was applied (−), or neutralized by the pretreatment with the anti-caprine IP-10 antibody (anti IP-10, 30 μg/ml), or with control rabbit IgG (control IgG). An *asterisk* indicates a significant difference ($p < 0.05$). For the results shown in *C*, *right panel*, and *D*, triplicate samples were examined for each treatment, and three independent experiments were performed.
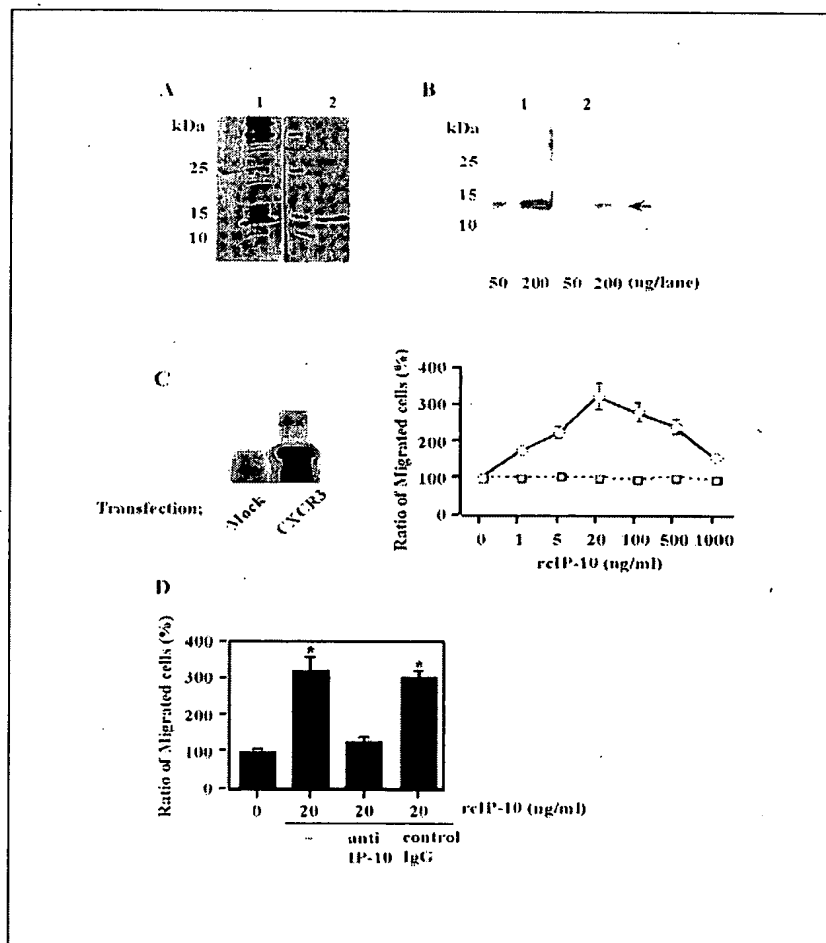
Biosciences). The protein was eluted from the column with a linear gradient of imidazole (20–500 mM), which was then dialyzed against PBS to remove imidazole.

200 μg of rcIP-10 proteins in PBS were mixed with Freund's complete adjuvant (Sigma) and injected sc into rabbits (*n* = 2). Recombinant proteins were injected two more times biweekly. A titer was checked 1 week after the last injection, and blood was collected on the following day. Anti-caprine IP-10 antibody was purified from antiserum using Hitrap Protein G column (Amersham Biosciences).

SDS-PAGE and Western Blot Analysis—After determining protein concentrations using protein assay kit II (Bio-Rad, Hercules, CA), culture media (3–8 μl, 10 μg of protein/lane, *n* = 3), concentrated uterine flushing media (10 μg of protein/lane, *n* = 3 for each day), or recombinant IP-10 (50 or 200 ng/lane) were subjected to Western blot analysis as described previously (7). In brief, protein samples were boiled for 5 min in the SDS sample buffer, electrophoresed on 10 or 15% SDS-PAGE gels under reducing conditions, and the gel was stained with Coomassie Blue. For Western blot analysis, proteins in the gel were transferred onto nitrocellulose membranes (Immobilon, Millipore, Bedford, MA). The membranes were blocked with Block Ace (Dainippon Pharmaceutical, Osaka, Japan) at room temperature for 1 h and then incubated with a rabbit polyclonal antibody to ovine IFN-τ (1:5000, a gift from Dr. Bazer), histidine tag (Sigma), or caprine IP-10 (1:1000) at 4 °C for 12 h. After incubation, membranes were washed three times in TBS-Tween 20, incubated with donkey anti-rabbit IgG-conjugated with horseradish peroxidases (Amersham Biosciences) at room temperature for 1 h, and washed three times in TBS-Tween 20. The bands were detected using ECL Western blotting detection reagents (Amersham Biosciences).

RT-PCR Analysis—Using Isogen (Nippon Gene, Tokyo, Japan), total RNAs were extracted from endometrial and conceptus tissues and cultured cells (*n* = 3 each). Total RNA samples were first reverse-transcribed with SuperScriptII (Invitrogen) and oligo(dT) primers (20 μl of reaction volume). Amounts of CXCR3, XCR1 and the integrin receptor mRNAs were determined from PCR amplification using oligonucleotide primers (Table I). Each reaction consisting of primer pairs was run with

RT template (1 μl) and AmpliTaq Gold (0.625 units; Roche Applied Science) in a final volume of 25 μl. All PCR reactions consisted of 40 cycles at 94 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min, followed by the final extension at 72 °C for 12 min. Following agarose gel electrophoresis and visualization with ethidium bromide, PCR products were quantified by using an image analysis system (ATTO Corporation, Tokyo, Japan) equipped with the Quantity One (v3.0 software; PDI, Inc., Huntington Station, NY).

Northern Blot Analysis—Northern blot analysis was performed as described previously (7). DIG-labeled cRNA probes were generated from IP-10 and CXCR3 cDNA plasmids by using T7 or SP6 RNA polymerase (Promega). Total RNAs were used for the determination of IP-10 mRNA whereas poly(A)+ RNA was used to examine CXCR3 mRNA. Poly(A)+ RNA was obtained from total RNA that had been isolated from PBMCs using Oligotex-dT30 (TaKaRa) according to the manufacturer's instructions. Total RNAs (20 μg/lane, *n* = 3 each) or poly (A)+ RNAs (2 μg/lane, *n* = 3 each) were separated by electrophoresis and transferred to a nylon membrane (Biodyne-B; Pall Corporation). The membrane was hybridized with cRNA probe at 63 °C for 12 h. After hybridization, the signals were detected using the DIG detection systems (7).

Immunofluorescence—Frozen conceptus tissues sectioned (10 μm, *n* = 3 for each day examined) were mounted onto silan-coated slides and fixed in acetone. Nonspecific binding was blocked by a treatment with Block Ace at room temperature for 1 h, and slides were then incubated with a mouse monoclonal antibody to human CXCR3 (10 μg/ml, R&D Systems Inc., Minneapolis, MN) or normal mouse IgG (Sigma) at 4 °C for 12 h. After the primary antibody incubation, slides were treated with fluorescein isothiocyanate-conjugated goat anti-mouse IgG (15 μg/ml, Jackson ImmunoResearch Laboratories Inc., West Grove, PA) at room temperature for 1 h. Nuclei were stained with propidium iodide (2 μg/ml, Sigma). The slides were examined and digital images were captured on a confocal laser scanning microscope (FV300, OLYMPUS, Tokyo, Japan).

Protein Biotinylation and Detection—Recombinant cIP-10 protein was biotinylated by EZ-link biotinylation reagent (Pierce). As a control,

FIG. 2. **Expression of IP-10 in the early pregnant uterus.** *A,* presence of IFN-τ in the culture media (10 μg proteins/lane) derived from days 14, 17, and 20 goat conceptuses was examined by Western blot analysis. *B,* Northern blots showing endometrial IP-10 mRNA. RNA (20 μg/lane) from the conceptus (day 17, *Con*) and endometrium (D14, D17, and D20) of pregnant goats (*left panel*), and endometrial explants (*right panel*) of cyclic goats (day 14) that had been stimulated with rcIFN-τ for 24 h were electrophoresed on a 1.2% agarose gel. *C, left,* presence of IP-10 protein in the uterine flushing media (10 μg proteins/lane) from pregnant or cyclic goats was examined using Western blot analysis. *Right,* densitometric analysis of Western blots of IP-10. *Bars* represent LSM ± S.E. An *asterisk* indicates a significant difference ($p < 0.05$) when compared with the value from day 14 cyclic uteri. *D, in situ* hybridization analysis of IP-10 mRNA in the caprine uterus. *Panels a, b,* and *c* represent DIG-labeled antisense caprine IP-10 cRNA on day 14 cyclic, day 14 pregnant, and day 17 pregnant goats, respectively; *panel d* is sense IP-10 on day 17 pregnant goats. *le,* luminal epithelium; *st,* subepithelial stroma; *tr,* trophoblast. *Bar,* 50 μm. Triplicate samples were analyzed for each day, and three independent experiments were performed.
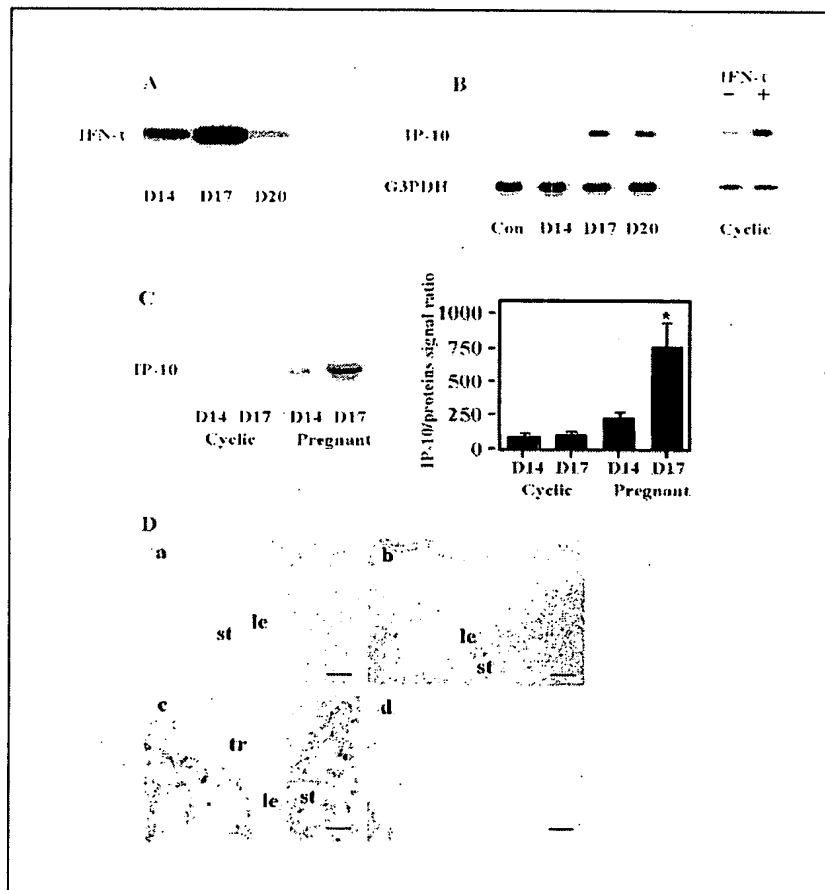
recombinant GST and recombinant caprine lymphotactin-GST protein[2] were also labeled with biotin. Proteins were incubated with 1 ml of 0.3 mg/ml EZ-link biotinylation reagent in PBS on ice for 2 h. After incubation, samples were dialyzed to remove the free biotin and measured their protein concentrations. For labeling efficiency conformation, biotinylated proteins (2 μg/lane) were resolved on 15% SDS-PAGE gels under reducing condition and visualized by streptavidin-horseradish peroxidases (Amersham Biosciences).

Frozen conceptus tissues sectioned (10 μm, $n = 3$ each) were mounted onto silan-coated slides and fixed in acetone. Slide sections were blocked with Block Ace at room temperature for 1 h and then incubated with biotinylated protein (100 μg/ml) at room temperature for 1 h. After incubation, slides were incubated with streptavidin-horseradish peroxidases (1:5000, Amersham Biosciences) at room temperature for 1 h. The biotinylated protein bound to the tissues was visualized with DAB solutions (Sigma). Tissue sections were then counterstained with hematoxylin.

Chemotaxis Assay—Migration of KU-1, HTS-1, or primary trophoblast cells was assessed in a 96 well-modified Boyden chamber (Neuro-Probe, Cabin John, MD) using polyvinylpyrrolidone-free polycarbonate membrane (5 μm pore size, NeuroProbe), which had been coated with 10 μg/ml bovine plasma fibronectin (Wako Junyaku, Osaka, Japan) for 2 h. The assay was performed as described (7). In brief, DMEM-0.1% bovine serum albumin (without phenol red or fetal calf serum) supplemented with indicated concentrations of rcIP-10 was added to the bottom wells of the chemotaxis chamber, whereas cells (5×10^6 cells/ml) in DMEM, 0.1% bovine serum albumin (without phenol red and fetal calf serum) were added to the top wells of the chamber. After the chambers were incubated at 37 °C and 5% CO_2 atmosphere for 2 h, the membranes were removed, washed with PBS, fixed, and stained with Dif-Quick (Kokusai Shiyaku, Kobe, Japan). The number of cells that migrated to the lower surface was microscopically counted at six randomly chosen high power fields. For the blocking experiments, before addition to the bottom chamber, rcIP-10 was preincubated at 37 °C for 1 h with 30 μg/ml of anti-IP-10 antibody or control rabbit IgG (Sigma).

Three replicates were performed for each treatment, and three independent experiments were done for each treatment.

Adhesion Assay—24-well plates were coated with type I collagen (Nitta gelatin) or fibronectin at a concentration of 10 μg/ml at room temperature for 2 h, or plated with caprine epithelial cells. Following three washes with PBS, the plates were blocked with 1% bovine serum albumin at room temperature for 30 min. HTS-1 or primary trophoblast cells were labeled with the intracellular fluorescent dye, 4 μM calsein-AM (Molecular Probes Inc., Eugene, OR) at 37 °C for 30 min. Following three washes with PBS, cells were incubated with the indicated concentrations of rcIP-10 at 37 °C for 1 h, and the cells were then added to each well, and the plates were incubated at 37 °C for 1 h. After incubation, unbound cells were removed by three washes with PBS, and the remaining cells were treated with 1% Triton X-100, 10% ethanol in PBS. Fluorescence of cells was measured using a fluorescence reader (excitation filter 485 nm and emission filter 535 nm) (ARVO™ SX 1420 Multilabel Counter, PerkinElmer Life Sciences Inc.). For the blocking experiments, rcIP-10 protein had been preincubated at 37 °C for 1 h with 30 μg/ml of anti-IP-10 antibody or control rabbit IgG (Sigma). To further investigate the involvement of IP-10 on cell adhesion to fibronectin, the Gly-Arg-Gly-Asp-Ser-Pro-Lys (GRGDSPK, Sigma) synthetic peptide at a concentration of 50 mM, its control, Arg-Gly-Glu-Ser (RGES, Sigma), or 5 mM EDTA was preincubated with cells and rcIP-10 protein. Three replicates were examined for each treatment, and three independent experiments were performed for each substrate and treatment.

Statistical Analysis—Measurements of optical density (Western blot) and light intensity (RT-PCR) were subjected to least squares (LS) ANOVA, which employed the general linear models procedures of the Statistical Analysis System (version 6.0; SAS Institute, Cary, NC). The light intensity from G3PDH PCR products was used as covariates for RT-PCR analyses. In chemotaxis assays, the number of cells migrated or attached with a treatment was calculated as the number relative to the cells without any treatment, which was then analyzed statistically as aforementioned. The model used in the LS-ANOVA included treatment and replicate as sources of variation. The least square means (LSMs) and S.E. illustrated in the figures were derived from this analysis.
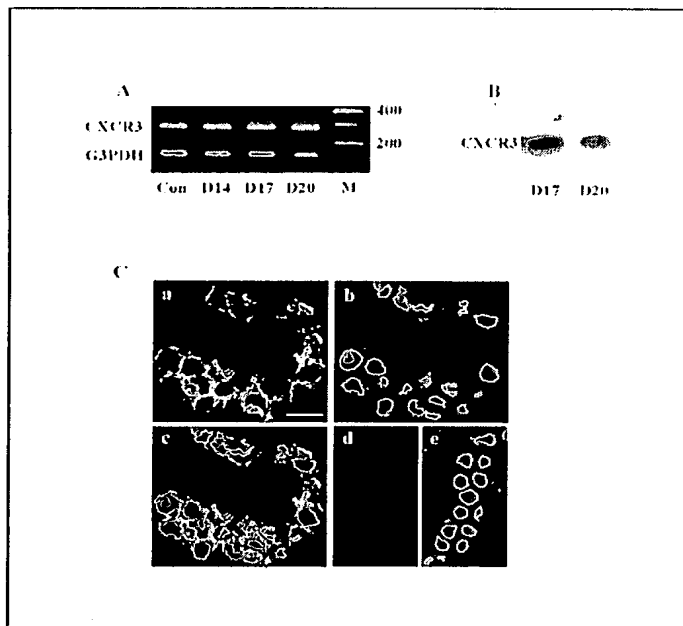
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[2] K. Imakawa, unpublished data.

FIG. 3. **Expression and cellular localization of CXCR3 in the caprine conceptuses.** *A,* levels of CXCR3 mRNA in the pregnant endometrium (D14, D17, and D20) and day 17 conceptuses (*Con*) were examined using RT-PCR. *B,* presence of CXCR3 mRNA in the caprine conceptus was confirmed by Northern blot analysis. For *A* and *B,* duplicate samples were analyzed for each day, and three independent experiments were performed for each day. *C,* immunofluorescence microscopy was performed on day 17 conceptuses using anti-human CXCR3 monoclonal antibody (*a*) or normal mouse IgG as negative control (*d*), and nuclei stained with propidium iodide were shown in the same field (*b* and *e*). *c,* combined fluorescence image resulting from anti-CXCR3 and propidium iodide was shown. *Scale bar,* 100 μm. Three independent experiments were performed for each treatment.

RESULTS

Preparation of rcIP-10 Protein and the Antibody—Recombinant cIP-10 (rcIP-10) was expressed in *E. coli* transformed with pET-14b-cIP-10 plasmid, purified using His tag systems, and confirmed by SDS-PAGE (Fig. 1*A*). Antibody to rcIP-10, purified from the antiserum using Hitrap protein G column, detected the specific band at ~14 kDa on Western blot analysis (Fig. 1*B*). To check the biological activity of rcIP-10, *in vitro* chemotaxis assay was also carried out: bovine B-cell line, KU-1, transiently transfected with the caprine CXCR3 cDNA was used as target cells. CXCR3 mRNA expression on the transfected cells was examined by Northern blot analysis (Fig. 1*C*, *left panel*). The chemotaxis assay demonstrated that CXCR3 transfectants consistently responded to 1–20 ng/ml rcIP-10, but their responses declined at higher rcIP-10 concentrations, resulting in a characteristic bell-shaped dose response curve (Fig. 1*C*, *right panel*). Moreover, immunoneutralization experiments revealed that an anti-rcIP-10 antibody reduced the chemotactic activity of rcIP-10 (Fig. 1*D*).

Expression of IP-10 within Goat Uterus during Early Pregnancy—The production of IFN-τ protein by caprine conceptuses was confirmed in the uterine flushing media and was the highest on day 17 of pregnancy (Fig. 2*A*). Northern blot analysis revealed that IP-10 mRNA level in the caprine endometrium began to increase from day 17 of pregnancy, and that the conceptus factor IFN-τ stimulated IP-10 mRNA level by day 14 cyclic endometrium *in vitro* (Fig. 2*B*). IP-10 protein was detected in the uterine flushing media, adjusted to 10 μg proteins/lane, from days 14 and 17 cyclic and pregnant animals (*n* = 3 for each day examined) (Fig. 2*C*). It appeared that the uterine flushing media from day 17 pregnant animals possessed more

IP-10 than those of other days examined. *In situ* hybridization revealed that IP-10 mRNA was observed in the subepithelial stroma regions of pregnant endometrium, and the intensity was greater in day 17 pregnant endometrium than that in day 14 cyclic or pregnant endometrium (Fig. 2*D*).

Functional Expression of CXCR3 Receptor in Trophoblast Cells—The expression of CXCR3 mRNA was observed in days 14, 17, and 20 caprine endometria (*n* = 3) and day 17 conceptus tissues (*n* = 3) during early pregnancy (Fig. 3*A*). Further confirmation of its expression in days 17 and 20 conceptuses (2 μg poly(A)+ RNA/lane, *n* = 3 for each day) was obtained by Northern blot analysis (Fig. 3*B*). To define the cellular origin of CXCR3 receptor in caprine conceptus tissues, immunofluorescence analysis was performed using frozen conceptuses and anti-CXCR3 antibody. A patchy fluorescence associated with the trophoblast layer was found and the localization was clearly different from nuclei (Fig. 3*C*). A dull autofluorescence was found on the trophoblast when the control mouse immunoglobulin was used.

To obtain more evidence on the significance of CXCR3 expression in the trophoblast cells, binding experiment of rcIP-10 protein to caprine trophoblast cells was performed. Recombinant cIP-10 protein along with recombinant GST and lymphotactin-GST were biotinylated, and the efficiency of biotinylation was confirmed (Fig. 4*A*). Lymphotactin belongs to the C chemokine family, and its expression, similar to IP-10, was observed in the goat endometrium during early pregnancy.[2] However, unlike CXCR3, the expression of lymphotactin receptor, XCR1, mRNA was not detected in the conceptus (Fig. 4*B*). For these reasons, lymphotactin/XCR was used as a negative control. The presence of CXCR3 was observed in the trophoblast cells incubated with only biotinylated rcIP-10 but not with biotinylated GST or lymphotactin-GST (Fig. 4*C*).

Recombinant cIP-10 Stimulation on the Migration of CXCR3-expressing Trophoblast Cells—It was suspected that IP-10 from the endometrium might interact with trophoblast cells expressing CXCR3 during early pregnancy. To study whether trophoblast cell migration was affected by endometrial IP-10 expression, *in vitro* chemotaxis assay was performed. Initially, trophoblast clone HTS-1 cells, which do not exhibit CXCR3 expression, were subjected to the chemotaxis study. HTS-1 transfected with the empty plasmid did not respond to rcIP-10 protein (Fig. 5*A*, *left panel*), however, HTS-1 transfected with CXCR3 consistently responded to 1–20 ng/ml rcIP-10, but their responses declined as rcIP-10 concentrations increased, resulting in a characteristic bell-shaped dose response curve (Fig. 5*A*, *right panel*). Moreover, immunoneutralization experiments revealed that an anti-rcIP-10 antibody reduced the chemotactic activity of rcIP-10 (Fig. 5*B*). In the next step, primary trophoblast cells expressing CXCR3 on day 17 of pregnancy were subjected to the chemotaxis assay. The migration of trophoblast cells was stimulated by rcIP-10 protein, and the chemotaxis activity was neutralized with the use of an anti-IP-10 antibody (Fig. 5*C*).

Effect of rcIP-10 on the Adhesion of Trophoblast Cells to Fibronectin and Endometrial Epithelial Cells—In addition to the migration activity of trophoblast cells to IP-10, adhesion assay was performed to determine whether IP-10 could increase the adhesion activity of trophoblast cells. Primary trophoblast cells bound to the plates coated with or without collagen I or fibronectin, but the binding rate of rcIP-10-stimulated cells to the fibronectin-coated plates was considerably higher than that of others (Fig. 6*A*). In the blocking experiment, rcIP-10 enhanced the adhesion of the trophoblast cells to fibronectin, which was inhibited by the anti-IP-10 antibody (Fig. 6*B*). The RGD peptide is known to inhibit cell
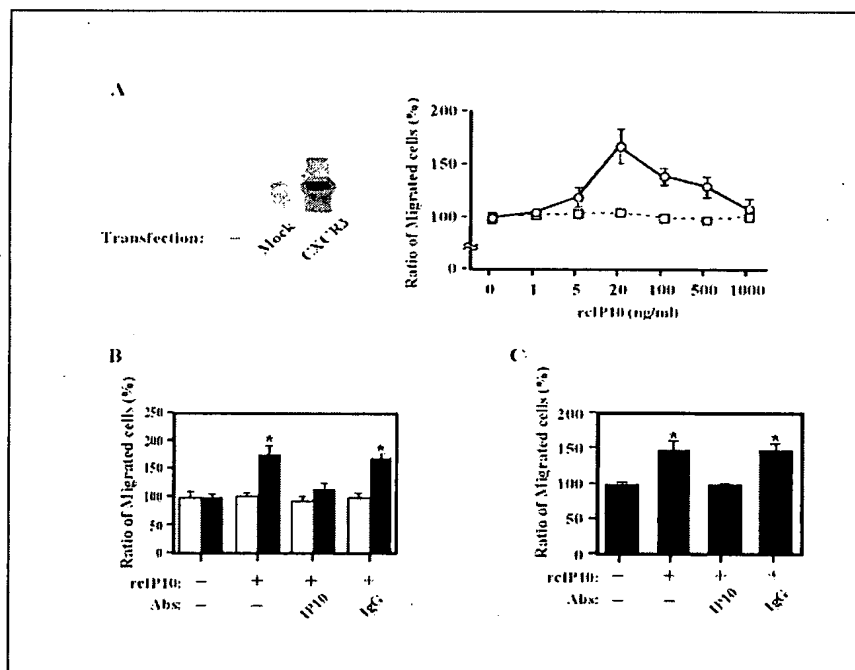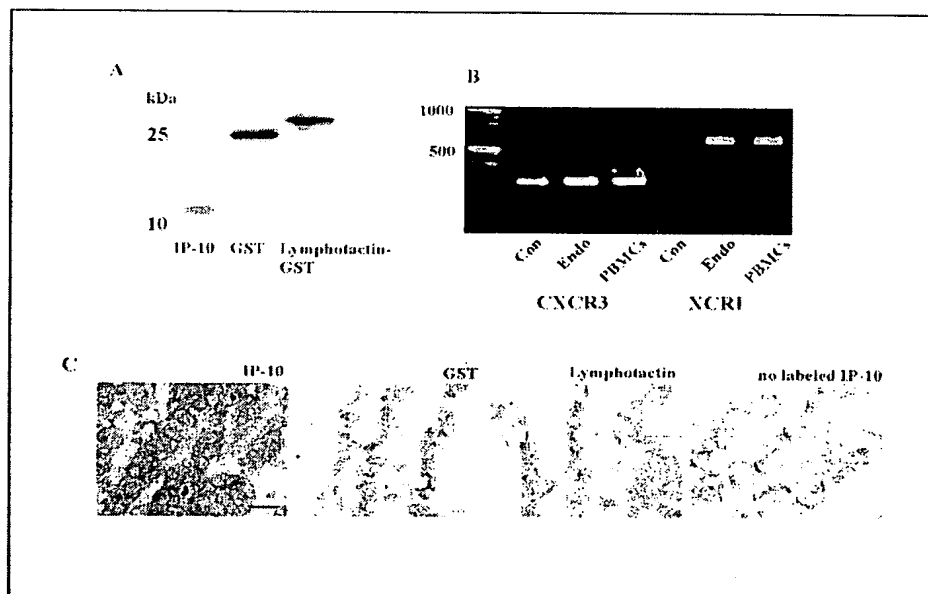
FIG. 5. **Stimulation of rcIP-10 on the migration of CXCR3-expressing conceptus cells.** *A*, *left*, Northern blot analysis of CXCR3 mRNA. RNA was extracted from HTS-1 cells (−) or those that had been transiently transfected with parental pcDNA3.1 *(Mock)* or pcDNA3.1-caprine CXCR3 *(CXCR3)*. *Right*, biological activity of rcIP-10 to HTS-1 cells that had been transfected *(circle)* or not transfected *(square)* with CXCR3 expression plasmid was tested using chemotaxis assay. *B*, effect of rcIP-10 on migratory activity of HTS-1 cells that had been transfected *(black bar)* or not transfected *(white bar)* with the CXCR3 construct was examined. These cells were treated (+) or not treated (−) with rcIP-10 (20 ng/ml), which had been pretreated with the anti-caprine IP-10 antibody (IP-10, 30 μg/ml) or control rabbit IgG (IgG, 30 μg/ml). Ratio of migrated cells was calculated as the number of migrated cells treated with rcIP-10 divided by the number of migrated cells without the rcIP-10 treatment. An *asterisk* indicates a significant difference ($p < 0.05$). *C*, effect of rcIP-10 on migratory activity of primary caprine trophoblast cells on day 17 of pregnancy was examined. For the blocking experiments, rcIP-10 (20 ng/ml) was preincubated with 30 μg/ml of anti-IP-10 antibody (IP-10) or normal rabbit IgG (IgG), which was then subjected to chemotaxis assay. *Bars* represent LSM ± S.E. An *asterisk* indicates a significant difference ($p < 0.05$). Three replicates were performed for each treatment, and three independent experiments were performed for each treatment.

adhesion occurring through RGD sites of fibronectin whereas the RGE peptide serves as an inactive control. RGD-treated cells, not RGE, decreased the adhesion of rcIP-10-stimulated cells to fibronectin (Fig. 6B). EDTA, known as an inhibitor of integrin signaling, also decreased the adhesion of trophoblast cells to fibronectin (Fig. 6B).

To obtain further evidence on IP-10-CXCR3 interactions,

HTS-1 cells transfected with or without CXCR3 cDNA were examined for adhesive activity. In HTS-1 cells transfected with CXCR3, but not without CXCR3, the adhesion of cells to fibronectin was stimulated by rcIP-10 protein and inhibited by the use of anti-IP-10 antibody in the blocking experiment (Fig. 6C). The addition of RGD peptides and EDTA abolished the adhesion of CXCR3-transfected cells to fibronectin (Fig. 6C).
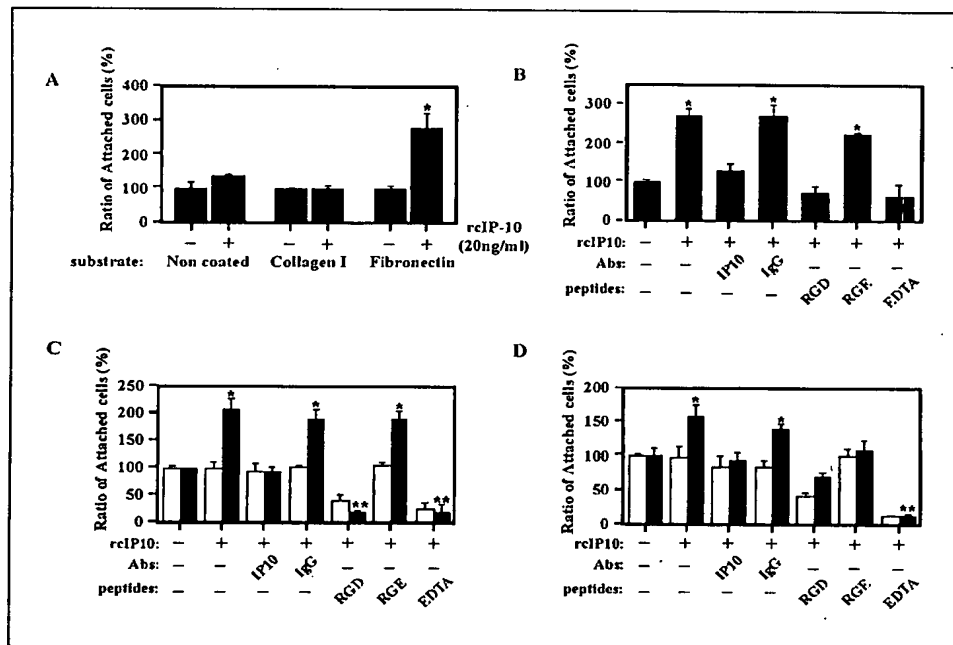
Regulation of Trophoblast Cell Migration and Adhesion by IP-10

FIG. 6. **Promotion of rcIP-10 on the adhesion of caprine trophoblast cells to fibronectin or endometrial epithelial cells.** *A*, adhesions of primary caprine trophoblast cells (day 17) stimulated (+) or not stimulated (−) with rcIP-10 (20 ng/ml) to the chamber coated with collagen I, fibronectin or non-substrate (non-coated) were assessed by using adhesion assay. *B*, effect of rcIP-10 (20 ng/ml) on the attachment of primary caprine trophoblast cells (day 17) to fibronectin was also assessed using the adhesion assay. Blocking experiment was performed using the pretreatment of rcIP-10 (20 ng/ml) with anti-caprine IP-10 antibody (IP-10, 30 μg/ml) or normal rabbit IgG (30 μg/ml), which was then added to the cells. The remaining treatments, 50 mM Arg-Gly-Asp (RGD) peptide, 50 mM Arg-Gly-Glu (RGE) peptide, or 5 mM EDTA, were applied to the trophoblast cells, which were then subjected to chemotaxis assay. *C*, HTS-1 transiently transfected with pcDNA3.1-caprine CXCR3 (*solid column*) or parental pcDNA3.1 (*open column*) were subjected to the fibronectin adhesion assay. Treatments with 20 ng/ml rcIP-10, 30 μg/ml of anti-IP-10 antibody (IP-10) or normal rabbit IgG (*IgG*), 50 mM RGD, or 50 mM RGE peptides, or 5 mM EDTA were applied as aforementioned. *D*, HTS-1 cells transiently transfected with pcDNA3.1-caprine CXCR3 (*solid column*) or parental pcDNA3.1 (*open column*) were subjected to the adhesion assay with endometrial epithelial cells. *Bars* represent LSM ± S.E. and an *asterisk*(s) indicates a significant difference (*p* < 0.05). Triplicate samples were examined for each treatment, and three independent experiments were performed.

Further decline of adhesion activity exhibited with RGD and EDTA treatments resulted from the observation in which cell adhesion occurred even in mock-transfected cells, and from the definition in which the degree of nonspecific adhesion was considered as 100% of attached cells. In this study, nonspecific and CXCR3 transfected cells to fibronectin were further inhibited by RGD or EDTA treatment. These results indicated that HTS-1 transfected with CXCR3 cDNA could reflect primary trophoblast cells in both cell adhesion and migration models.

Furthermore, whether trophoblast cells activated by IP-10 could bind to the endometrial epithelial cells was investigated. Fig. 6*D* shows that the adhesion of CXCR3 expressing HTS-1 to primary epithelial cells from the caprine endometrium was increased by rcIP-10 protein, and the adhesive activity was inhibited by the use of the IP-10 antibody in the neutralizing experiment. EDTA-treated trophoblast cells lost the adhesion activity to epithelial cells. Interestingly, the adhesion activity of RGD peptide-treated cells was reduced but the activity was higher than the EDTA-treated ones (Fig. 6*D*).

Expression of Integrin Subunits in Trophoblast Cells Stimulated by IP-10—To define which integrin subunit was involved in the IP-10-induced trophoblast cell adhesion to fibronectin, the expression of integrin subunits, major candidates to bind fibronectin, was examined in HTS-1 cells stimulated by rcIP-10. Transcripts for integrin subunits, α_5, α_V, β_1, β_3, and β_5, were detected by using RT-PCR. The expression of integrin α_5, α_V, and β_3 subunits mRNA in trophoblast cells were stimulated by rcIP-10, and the stimulation was diminished by the neutralization with the anti-IP-10 antibody (Fig. 7). The expression of integrin β_1 and β_5 subunits mRNA was not influenced by rcIP-10.

DISCUSSION

Recently, the presence of several chemokines and/or their receptors in the conceptus and maternal endometrium has been recognized during early pregnancy. These chemokines are suspected to play a role in the processes of implantation, but a definitive role that chemokines play during early pregnancy has not been identified. In the present study, we detected IP-10, mRNA and protein, in the subepithelial stroma and uterine lumen, respectively, and its receptor CXCR3 localized in the conceptus is functioning during early gestation. These results indicate that other than the regulation of immune cell distribution, a chemokine present in the pregnant uterus plays an important role in the regulation of trophoblast cells for their migration and adhesion to the endometrial epithelial cells. This work therefore represents the demonstration of a trophoblast cell mobilization in the early stage of conceptus implantation to the maternal endometrium.

The expression of CXCR3 mRNA in the conceptus tissues was detected using RT-PCR and Northern blot analysis. It is known that conceptus tissues during peri-implantation period consist of several different cell types, to which maternal leukocytes bind (27), therefore, the determination of the cellular origin of CXCR3 mRNA was required. The expression of a leukocyte marker CD45 mRNA was detected in RNA samples extracted from conceptuses, but its level was lower than that in blood leukocyte samples (data not shown). However, immunofluorescent and ligand binding analyses revealed that CXCR3 receptors were localized in the trophoblast cells but not in leukocytes, suggesting that the caprine trophoblast had the ability to respond to IP-10 produced from the endometrium.

FIG. 7. **Expression of integrin subunits in the caprine trophoblast cells stimulated by rcIP-10.** *Upper*, RT-PCR analysis of expressions of integrin α_5, α_V, β_1, β_3, and β_5 subunits, and G3PDH mRNAs. Recombinant cIP-10 (20 ng/ml) pretreated (IP-10 + Abs) or not pretreated (IP-10) with anti-IP-10 antibody (30 μg/ml) was applied to HTS-1 cells that had been transiently transfected with pcDNA3.1-caprine CXCR3. *Lower*, densitometric analysis of PCR products. *Bars* represent LSM ± S.E., and differences in integrin mRNA levels among treatments were shown with a *superscript*.
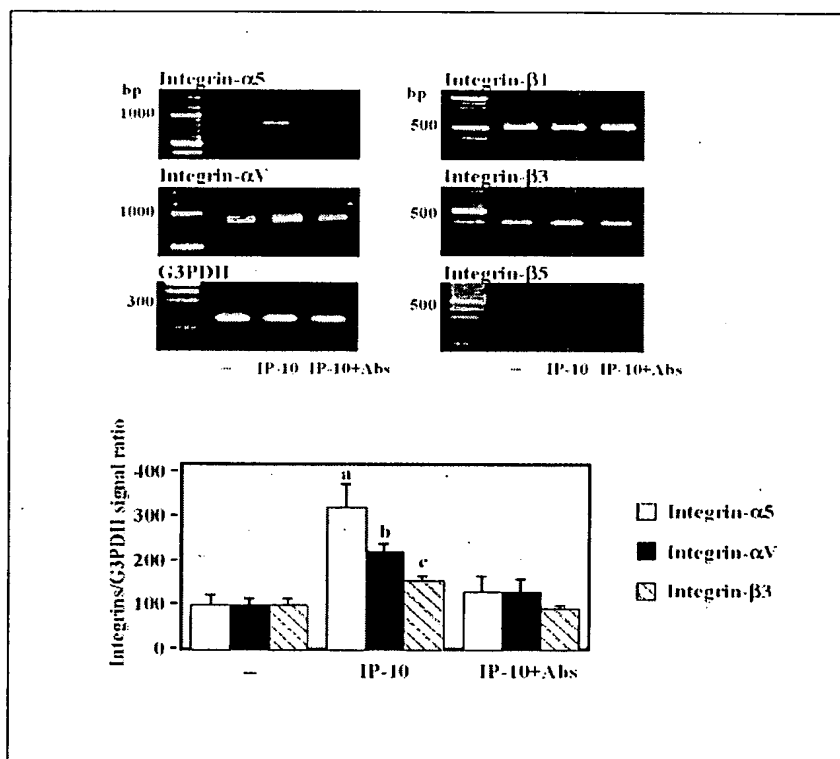
Northern blot analysis revealed that the expression of CXCR3 mRNA in the conceptus tissues declined from day 17 to day 20 of pregnancy. These results suggest the possibility that trophoblastic CXCR3 was regulated by a factor(s) other than endometrial IP-10 during early pregnancy. It was reported that the removal of IL-2 from CXCR3+ T cell cultures results in rapid loss of cell surface CXCR3 (34), and recombinant IFN-γ could stimulate the expression of chemokine receptor, CCR3, in the human trophoblast cells (26). Other than these observations, however, the regulation of chemokine receptor expression has not been definitively elucidated. Therefore, further investigation is required to determine the regulation of CXCR3 expression in the caprine trophoblast cells.

Implantation is completed through a number of steps in which the trophoblast cells undergo a series of distinct interactions with maternal endometrium. It is initiated by close proximity between trophoblast and endometrial epithelial cells, promoted by not only closure of the uterine lumen but also activation of blastocyst migration. In chemokines and their receptor interactions, cells possessing a chemokine receptor move into the direction of increasing concentrations of a chemokine, which typically is a soluble molecule that could diffuse away from the site of production where its concentration is highest (35, 36). In this study, IP-10 mRNA was not found in the conceptus, but was detected in the subepithelial stroma, and the protein was present in the uterine flushing media, providing evidence that IP-10 could have diffused into the pregnant uterine lumen. These results and the observation in which migration of isolated primary trophoblast cells was regulated by IP-10 suggest that endometrial IP-10 establishes its concentration gradient in the pregnant uterus, and trophoblast cells could sense concentration differences and migrate toward the endometrium.

In ruminant ungulates, elongated conceptus occupies almost entire uterine horn during the initial attachment stage; days 17–18 and days 18–19 pregnancy in sheep and goats, respectively. This association or close proximity between two organ-isms, which could be regulated through the IP-10/CXCR3 system, may increase chances of conceptus attachment to the caruncular regions of the uterus. It has been noted that apical epithelial surfaces are normally non-adhesive, yet during implantation, the transition to pre-receptive state such as a decline in mucin Muc-1 is required in these cells (5, 37, 38). Initial adhesion of the trophoblast cells involves physical adhesion and may interact with the appropriate ECM on the endometrium. In the present investigation, trophoblast cell adhesion to non-, collagen I- or fibronectin-coated plates was observed, and the adhesion to the fibronectin-coated plates was most stimulated by the addition of IP-10. Fibronectin belongs to a member of ECM molecules identified at the conceptus-endometrium interface, which is capable of serving as a ligand for the receptor, integrin family. Fibronectin is also implicated in murine trophoblast outgrowth and adhesion processes, both of which could be blocked with RGD-containing peptides *in vivo* (39) and *in vitro* (40). The blocking with RGD peptides abolished the IP-10 stimulated adhesion activity of trophoblast cells to fibronectin, and EDTA, an inhibitor of integrin signaling, also abolished the adhesion activity. These results were further confirmed by the observations that trophoblast cells stimulated by IP-10 increased the adhesion to endometrial epithelial cells, which was reduced by the treatment with the RGD peptide. These results indicated that IP-10 could stimulate the expression or activation of integrins on the trophoblast cell surface and promote the trophoblast cell adhesion to one of ECM, fibronectin.

In this study, IP-10 induced the expression of integrin α_5, α_V, and β_3 subunits in caprine trophoblast cells. Our results agree with previous reports that the presence of $\alpha_5\beta_1$, $\alpha_V\beta_1$, $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrins on the trophoblast cell surface is required for successful interaction between the trophoblast and endometrium (4, 41–43). In addition, $\alpha_5\beta_1$, $\alpha_V\beta_1$, $\alpha_V\beta_3$, and $\alpha_V\beta_5$ integrin isoforms recognize ECM ligands that contain the RGD sequence. Although the *in vivo* data are not available, these results from the *in vitro* study suggest that trophoblast cell

migration is regulated by endometrial IP-10, which could control the expression of some integrin isoforms, $\alpha_5\beta_1$, $\alpha_V\beta_1$, $\alpha_V\beta_3$, and $\alpha_V\beta_5$, on the apical surface, facilitating the adhesion of trophoblast cells to endometrial ECM ligands. Interestingly, the effect in RGD blocking of the trophoblast cell adhesion to the epithelial cells was lower than that to the fibronectin. These results suggest that although IP-10 could stimulate the adhesion of trophoblast cell to particular RGD-containing ECM, other adhesion molecule(s) was also present at the sites of interactions between trophoblast and epithelial cells *in utero*. A molecule, L-selectin, expressed on the human trophoblast cells recently described by Genbacev *et al.* (44), could also be playing a role in the establishment of initial attachment to the endometrium. Involvement of other adhesion molecule(s) predicted in this study might be L-selectin. Based on these observations, we propose that the production of endometrial chemokines, stimulated by a conceptus factor(s), is required for the expression of suitable adhesion molecules on trophoblast cells, resulting in the progression to successful implantation.

In conclusion, we identified a cell mobilization pathway potentially functioning in the early stage of embryonic implantation to the endometrium. For successful implantation in goats and other ruminant ungulates, the endometrial IP-10 and trophoblastic CXCR3 might regulate the trophoblast apposition and adhesion to the endometrium through the stimulation of integrin expression, recognizing the RGD-containing ECM, on the trophoblast cell surface. Since the initial apposition and adhesion of the conceptus to the endometrium occurs universally among mammals, the presence of IP-10 or other chemokines with similar functions and their receptors is prerequisite for engagement of the conceptus to the endometrium.

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